International Journal of Farming and Allied Sciences
Available online at www.ijfas.com
©2013 IJFAS Journal-2013-2-22/955-960
ISSN 2322-4134 ©2013 IJFAS

Evaluation of genetic diversity in different genotypes of (Medicago Sativa L.) using ISSR markers

Fatemeh Bolourchian[1*], Mohsen Farshadfar[2], Hooshmand Safari[3], Hooman Shirvani[2]

1. Department of Agriculture, Islamic Azad University, Kermanshah Branch, Kermanshah, Iran
2. Department of Agriculture, Payame Noor University, Iran
3. Agriculture and Natural Resources Research Center, Kermanshah, Iran

Corresponding author: Fatemeh Bolourchian

ABSTRACT: Alfalfa (Medicago sativa L.) is a widely grown legume and one of the most important forage species throughout the world. It yields forage of high nutritional quality and has positive influences on the environment. Genetic diversity based on different markers, plays a key role in breeding programs and one of the most important criteria for selection of Parents. In this study the genetic diversity among 19 genotypes of *Medicago sativa* was investigated using 8 ISSR primers. These primers could identify 37 loci. Primer IS5 showed the highest number of bonds(7) and primers IS1, IS9 and IS16 showed the lowest number of bonds(3). The Average percentage of polymorphism was 86% for primers used, that the lowest percentage of polymorphism was for IS14 and IS15 (50%) and IS5 (57.14%). Also it was 100% for other primers. The average of PIC for studied primers was 0.32 that highest rate of PIC belong to IS9 and IS13 and the primers IS5 and IS16 had the lowest. Also, cluster analysis and principal coordinate analysis using Dice similarity coefficients were calculated and dendrogram was drawn using the UPGMA for 19 genotypes. The coordinate analysis was performed using of the similarity matrix and confirmed the results of cluster analysis.

***Keywords*:** Alfalfa, Genetic diversity, Molecular marker, ISSR

INTRODUCTION

Medicago sativa is one of the most important forage crops in Iran growing in various regions of the country. Southwest Asia and possibly northern Iran are considered to be the place of its origin (Rezai, 1992). Alfalfa is autotetraploid (2n = 4x = 32), allogamous and a seed-propagated species. These factors contribute to the genetic complexity of alfalfa (Labombrada et al., 2000). It is the most cultivated forage legume, with about 32 million ha over the world. Its agronomical interest is based on its high protein content, suitable feeding value and favorable environmental impact (perennial and no nitrogen fertilizer required). Molecular markers, unlike from traditional phenotypic markers, detect variability and differences among and within cultivars directly at the DNA level. As they are independent of environmental factors, they enable a more precise determination of genetic relatedness. Different molecular marker types have been used to assess genetic diversity in alfalfa. Several studies have been conducted using molecular markers to assess the level of variation among perennial Medicago species and populations (Brummer et al., 1991; Echt et al., 1992; Yu and Pauls, 1993; kidwell et al., 1994) and one study used RAPD (random amplified polymorphic DNA) markers to study variation among annual species (Brummer et al., 1995). No one of these studies utilized SSR markers. To date, few studies have been conducted using SSR markars to assess the level of variation among perennial Medicago species and populations. (Diwan et al., 1997)

have been the first to develop SSR markers in Medicago. They have shown how SSR can be used to describe genetic diversity and to analyze the genetic relationships among genotypes in alfalfa. Recently a set of 107 SSRs identified in the EST data base of Medicago truvcatula was mapped in Medicago sativa (Julier et al., 2003) and can be used to perform genetic diversity. Other researchers, including(Megoni et al., 2000)of these markers to study the genetic relationship among ecotypes have used Medicago sativa crop. Some of this locus for survey structure distance and genetic diversity within populations in species M. truncatula were used (Bonnin and Ronfort, 2001). Inter Simple Sequence Repeat (ISSR) is a dominant molecular marker revealed in mass. ISSR has recently been developed as an anonymous, RAPD – like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes and circumvents the challenge of characterizing individual loci that other molecular approaches require. They are characterized by mono-, di- or multi - nucleotide repeats that have 4 -10 repeat units side-by-side. Extremely high variability combined with greater robustness in repeatability experiments and less prone to changing band patterns with changes in constituent or DNA concentration template make them superior to other readily available marker systems in investigations of genetic variation (Fang and Roose, 1997). The purpose of this study was to evaluate the genetic diversity of 19 genotypes of Medicago sativa using ISSR molecular markers.

MATERIALS AND METHODS

In this study, 19 genotypes of Medicago sativa species were used to carry out experiments. (Table1). Experiments were performed in the biotechnology laboratory of Agriculture and Natural Resources Research Center of Kermanshah, Iran.

Table1. Name and codes of genotypes

Number	Origin	Gene bank Code
1	ESFAHAN	ES-211
2	SHAROD	ES-027
3	ESFAHAN	Es-037
4	CODI	Es-199
5	YAZDY	Es-024
6	KERISARI	Es-054
7	FAO	KR-3003
8	TORBAT	Es-032
9	TEHRAN	Es-011
10	BIRJAND	Es-034
11	FLOWER	Es-083
12	CODI	Es-058
13	KERMAN	Es-036
14	ZARDSH1	Es-065
15	SIRJAN	Es-006
16	GORGAN	Es-050
17	ESFAHAN	Es-126
18	MASHAD	Es-031
19	unknown	G-19

ISSR Method

Total genomic DNA was extracted for young leaves of greenhouse-grown plants using a modified CTAB (Murry and Tompson, 1980) with modification described by(De la Rosa et al., 2002). Quality and quantity of extracted DNA were examined using 0.8% agarose gel. The compounds of polymerase chain reaction were carried out according to table 2.

Table 2. compounds of optimized ISSR reaction

To provide 20 µl	compounds of a sample
12.6 µl	Water distillated twice
2 µl	Buffer PCR (X10)
1.5 µl	$MgCl_2$ (50 mmol)
0.4 µl	Nucleotides mixture (10 mmol)
1.2 µl	Primer (10 µmol)
0.3 µl	Tag polymerase
2 µl	DNA (10 ng)
20 µl	total

Template DNA was initially denatured at 92^{o}C for 5 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 30 seconds at 95^{o}C, primer annealing for 30 seconds at the temperature based on primer temperature (temperatures of annealing in this study was 50, 55 and 60 ^{o}C) and primer extension for 1 min at 72^{o}C. A final incubation for 5 min at 72^{o}C was performed to ensure that the primer extension reaction proceeded to completion. The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using TBE buffer. The gels were put in the Ethidium bromide for 30-45 min and visualized by gel document.

Statistical analysis

ISSR bands were treated as binary characters and coded accordingly (presence =1, absence = 0). Number of bands scored, Number of polymorphic bands, Percentage of polymorphic bands were calculated for each primers and each genotypes. Polymorphism information content was measured for each primer (Anderson et al., 1993). Cluster analysis, similarity matrix and principal coordinate analysis axis were carried out for 19 genotypes using Darwin and NTSYS.

$$PIC = 1 - \sum_{i=1}^{n} PI^{2}$$

RESULTS AND DISCUSSION

RESULT

In this study the genetic diversity among 19 genotypes of *medicago sativa* was investigated using 8 ISSR primers. These primers could identify 37 loci which 8 bands of them were polymorphic. Primer IS5 showed the highest number of bands (7) and primers IS1, IS9 and IS16 showed the lowest number of bands (3) (figure 1).

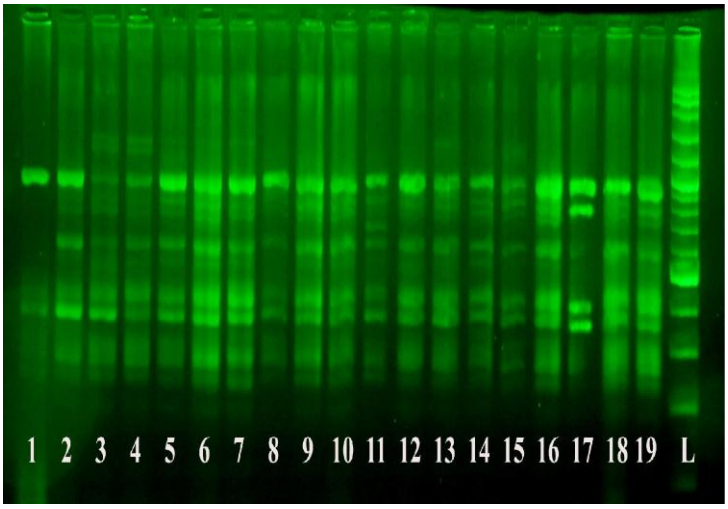

Figure 1. band pattern of 19 genotypes alfalfa using Primers IS3

Average percentage of polymorphism was equal to 86%, the lowest percentage of polymorphism was 50% and belonged to primers IS14 and 1S15 and the highest was 100% and belonged to primers IS1, IS3, IS9, IS13 and IS16 respectively. The average of bands for each primer was 4.62 for 19 genotypes that genotypes 6(Es-054) and 16(Es-050) had the most and genotypes 3(Es-037), 4 (Es-199) and 8 (Es-032) had the lowest band. The results of used primers have been showed in table 3. The most amount of PIC related to IS 9 and IS13 that these primers could determine genetic distance much better than other primers so it can be able to use of these primers for analysis of other genotypes of alfalfa germplasm in future research. The primers IS5 and IS16 with lowest PIC didn't have good ability to separate genotypes.

Table 3. ISSR primers used in this study and some summary results

ISSR code	Primer sequence	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands(PPB)	PIC
IS1	5'- ACACACACACACACAC YC-3'	3	3	100%	0.27
IS3	5'- GAGAGAGAGAGAGAGAYC-3'	5	5	100%	0.35
IS5	5'- AGAGAGAGAGAGAGAGC-3'	7	4	57.14%	0.22
IS9	5'- CTCT CT CTCT CTCTCT G-3'	3	3	100%	0.46
IS13	5'- AGAGAGAGAGAGAGAGYT-3'	6	6	100%	0.47
IS14	5'- GACAGACAGACAGACA -3'	4	2	50%	0.24
IS15	5'- GGATGGATGGATGGAT -3'	6	3	50%	0.36
IS16	5'-DBDACACACACACACACA-3'	3	3	100%	0.22
Average		4.62	3.62	86%	0.32

Similarity matrix

Similarity matrix based on Dice coefficient (Table 4), showed the highest genetic similarity between genotypes 18(Es-031) and 19(G-19) from group 3 with a similarity coefficient 0.983 and the lowest similarity belong to genotype 1(ES-211) from group 2 with genotype15 (Es-006) from group 3 with similarity coefficient 0.705.

Table 4. Similarity matrix for studying genotypes based on Dice's coefficient

Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1																		
2	0.87	1																	
3	0.85	0.85	1																
4	0.81	0.85	0.82	1															
5	0.78	0.82	0.79	0.75	1														
6	0.76	0.86	0.73	0.73	0.78	1													
7	0.75	0.89	0.79	0.75	0.81	0.84	1												
8	0.81	0.77	0.73	0.82	0.75	0.73	0.72	1											
9	0.82	0.89	0.75	0.75	0.74	0.88	0.81	0.72	1										
10	0.82	0.81	0.70	0.83	0.68	0.74	0.80	0.78	0.80	1									
11	0.82	0.82	0.72	0.75	0.81	0.88	0.84	0.72	0.81	0.80	1								
12	0.81	0.88	0.82	0.82	0.72	0.80	0.86	0.82	0.83	0.82	0.76	1							
13	0.75	0.85	0.75	0.72	0.81	0.91	0.87	0.68	0.84	0.76	0.87	0.83	1						
14	0.77	0.81	0.69	0.82	0.75	0.77	0.86	0.78	0.76	0.90	0.86	0.81	0.76	1					
15	0.71	0.82	0.71	0.63	0.77	0.78	0.81	0.67	0.81	0.68	0.74	0.75	0.84	0.68	1				
16	0.79	0.92	0.76	0.76	0.81	0.91	0.91	0.76	0.88	0.77	0.88	0.83	0.88	0.83	0.85	1			
17	0.79	0.79	0.72	0.68	0.78	0.85	0.75	0.76	0.88	0.65	0.78	0.69	0.78	0.65	0.74	0.85	1		
18	0.78	0.85	0.75	0.82	0.73	0.84	0.87	0.82	0.87	0.87	0.80	0.89	0.80	0.89	0.73	0.87	0.77	1	
19	0.76	0.83	0.73	0.81	0.75	0.83	0.89	0.81	0.85	0.85	0.82	0.88	0.79	0.91	0.71	0.86	0.76	0.98	1

Cluster analysis

Cluster analysis was performed based on Dice similarity coefficient and the genotypes studied was divided into three groups, the first group involve of four genotype 3(ES-037), 5(ES-024), 8(ES-032) and 17(ES-126) and the second group includes 5 genotype 1(ES-211), 4(ES-199), 10(ES-034), 11(ES-083) and 14(ES-065) the third group consisted of 9 genotypes 2(ES-027), 6(ES-054), 7(ES-3003), 9(ES-011), 12(ES-058), 13(ES-036), 15(ES-006), 16(ES-050), 18(ES-031) and 19(G19) respectively (Figure 2).

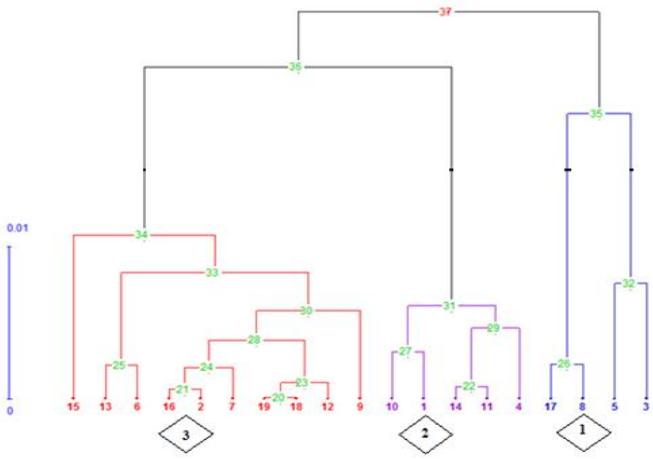

Figure 2. Dendrogram of cluster analysis for genotypes based Dice's coefficient by UPGMA.

Principal coordinate analysis

The coordinate's analysis was performed using of the similarity matrix and the result confirmed the result of Cluster analysis to a large extent and genotypes was divided to 3 groups (Figure 3).

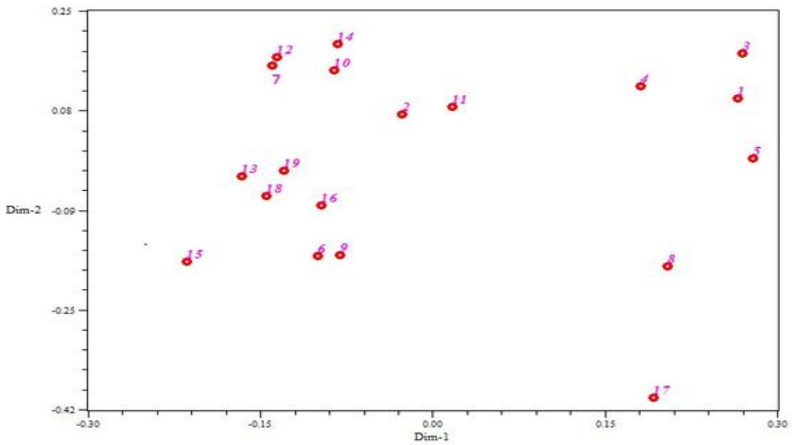

Figure 3. Scatter plot for genotypes based on two first axes from principal coordinate analysis

DISCUSSION

According to this study a Significant variation was observed among genotypes. The Average percentage of polymorphism was 86% that showed a good polymorphism between genotypes. Between 8 used primers, the primers IS1, IS3, IS9, IS13 showed 100% polymorphism that indicate high ability for survey molecular variation and high variation among genotypes. Also this study showed that it can be able to determine genetic pattern of Medicago genotypes to each other by ISSR method in a short time. Efficiency of ISSR primers were reported by

other researchers to determine of genetic diversity between and within different plant species (Habibi et al., 2012) Grouping of genotypes based on cluster analysis and principal coordinate analysis indicated that genetic variations do not agreement with the geographical distribution of genotypes. In survey genetic diversity within and between 19 variety and line Medicago sativa genotypes based on cluster analysis divided to different groups and showed that genetic diversity within variety and lines difference Medicago sativa more of between diversity (Musial et al., 2002).

REFERENCES

Anderson JA, Church JE, Autrique SD, Thanksley S, Sorrells ME. 1993. Optimizing parental selection for genetic linkage map. Journal of Genome. 36(1): 181-188.

Bonnin I and Ronfort J. 2001. Spatial effect and rare outcroosssing events in M. truncatula (Fabaceae). Journal of Molecular.

Brummer EC, Bouton JH, and Kochert G. 1991. Analysis of annual Medicago species using RAPD markers. Genome. 38:362- 367.

Brummer EC, Kochert G and Bouton JH. 1991. RFLP variation in diploid and tetraploid alfalfa. Theor. Appl. Genet. 83: 89-96.

De La Rosa R, James C and Tobutt KR. 2002. Isolation and characterization of polymorphic microsatellite in olive (Olea europaea L.) and their transferability to other genera in Oleaceae. Mol Ecol Notes 2: 265-267.

Diwan N, Bouton JH, Kochert G, Cregan PB. 2000. Mapping simple sequence repeats (SSR) DNA markers in diploid and tetraploid alfalfa. Journal of Theoritical and Applied Genetics. 101(1-2): 165-172.

Echt CS, Erdahl LA and Mccoy TJ. 1992. Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa. Genom, 35: 84-87.

Ecology. 10(6): 1371- 1384.

Fang DQ and Roose ML. 1997. Identification of closely related citrus cultivars with inter-simple sequence repeats markers. Theor. Appl.Genet. 95: 408-417.

Habibi B, Farshadfar M, Safari H. 2012. Evaluation of Genetic Diversity in 18 Genotypes of Alfalfa (Medicago Sativa) Using of Molecular ISSR Markers. International Journal of Agriculture and Crop Sciences 4(21): 1573-1578.

Juleir B, Flajolot S, Barre P, cardinet G and Sylvain S. 2003. Construction of two genetic linkage maps in cultivated tetraploid alfalfa (medicago sativa L.) populations. Plant Genet.Breed, 53: 401- 407.

Kidwell KK, Austin DF and Osborn TC. 1994. RFLP evaluation of nine medicago accessions representing the original germplasm sources of North American alfalfa cultivars. CropSci. 34: 230-236.

Labombrada P, Pupilli F, Arcioni S. 2000. Optimal population size for RFLP- assisted cultivar identification in alfalfa (Medicago sativa L.). Agronomie 20: 233–240.

McCoy TJ and Bingham ET. 1988. Cytology and cytogenetics of alfalfa, in Alfalfa and alfalfa improvement. Agronomy Monograph 29 (ed. A. A. Hanson, D. K. Bbarnes and R. R. Hill), Madison USA. pp. 737- 776.

Mengoni A, Gori A, Bazziacalupo M. 2000. Use of RA PD and microsatellite (SSR) variation to assess geneticrelationship among populations of tetraploid alfalfa (Medicago sativa). Journal of Plant Breeding, 119(4): 311-317.

Murry MG and Tompson WF. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Research 8: 4321-4325.

Musial JM, Basford KE, Girwin JA. 2002. Analysis of genetic diversity within Australian Lucerene cultivars and implication for future genetic improvement. Australian Journal of Agriculture Research, 53(6): 629-636.

Rezai A. 1992. Breeding Alfalfa. Tehran University Publication, Tehran.

Yu KF and Pauls KP. 1993. Rapid estimation of genetic relatedness among heterogeneous Population of Alfalfa by random amplification of bulked genomic DNA samples. Theor. Apppl. Genet. 86: 788- 794.